Inhibition of Enzymes by Metal Ion-Chelating Reagents

THEORY AND NEW GRAPHICAL METHODS OF STUDY

By WILLIAM G. BARDSLEY and ROBERT E. CHILDS

Department of Obstetrics and Gynaecology, University of Manchester, St. Mary's Hospital, Whitworth Park, Manchester M13 0JH, U.K.

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1. The mechanism of inhibition of enzymes by metal ion-chelating reagents is discussed and equations derived. 2. Two distinct mechanisms are postulated and graphical methods are given for differentiating between them. 3. Where the metal ion is actually removed from the enzyme to form a co-ordination complex in solution, a procedure is described for obtaining the stability constant for metal–enzyme interaction, the number of metal ions involved and the stoichiometry of metal ion–ligand interaction.

Despite the wide diversity of structural and functional types of metal ion–protein complexes encountered, metalloenzymes and metal-activated enzymes are usually distinguished on the basis of enzyme–metal ion affinity. A stability constant of 10^8 M^{-1} has been suggested as a dividing line (Mildvan, 1970).

Several experimental methods are available for studying the type of complex formed, its stoichiometry and mode of involvement in the catalytic process or substrate binding. Estimation of metal ion content during purification gives the total number of ions involved and their identity and this information can usually be supplemented by such techniques as dialysis and metal ion replacement or equilibrium dialysis (Hughes & Klotz, 1956). Physical methods have proved useful in studying the nature of any co-ordination complexes, the environment in which they occur and mode of involvement in the catalytic sequence (Mildvan, 1970; Mildvan & Cohn, 1970).

Kinetic techniques, involving titration of the enzyme with metal ion-chelating agents, have also proved valuable. For instance when 1,10-phenanthroline was added to yeast alcohol dehydrogenase an immediate inhibition was produced owing to ligand substituting into the co-ordination sphere of Zn^{2+} ions. This was found to be competitive between 1,10-phenanthroline and NAD^+ (Hoch & Vallee, 1956) but not competitive with respect to ethanol or acetaldehyde (Hoch *et al.*, 1958). Preincubation of enzyme and inhibitor gave time-dependent irreversible inhibition (Williams *et al.*, 1958) which was caused by the enzyme dissociating into four subunits as the four Zn^{2+} ions were removed from the enzyme to form co-ordination complexes in solution (Kägi & Vallee, 1960).

The present paper discusses the mathematical formulations needed to describe the inhibition by metal ion-chelating agents and suggests diagnostic

tests that can be applied to distinguish between alternative mechanisms of inhibition.

Theory

We consider two extreme cases; where the inhibitor merely substitutes ligands on the enzyme, the metal remaining attached to the enzyme and where the metal ion is completely removed by the inhibitor.

Type I mechanism: the chelating agent binds to the metal on the enzyme

It is believed that most complex-forming reactions of biologically important ions involving ligand displacement proceed by $\text{S}_{\text{N}}1$ outer-sphere mechanisms (Mildvan, 1970). As most metal ion-chelating agents used to inhibit enzymes are multidentate, this probably means that only one incoming ligand can displace the enzyme ligand since, if a second ligand species was inserted to form a chelate ring, the metal ion would not be held sufficiently strongly to the enzyme and would dissociate. For instance, a square-planar complex could still be bound to the enzyme if one bidentate ligand substituted into the co-ordination sphere but substitution of a second molecule of bidentate ligand could only occur if the metal ion dissociated from the enzyme. Now, if the enzyme gave linear double-reciprocal plots both in the presence and the absence of inhibitor (metal-chelating agent), we would expect eqn. (1) to apply where $f[\text{I}]$ and $g[\text{I}]$ are polynomials in inhibitor concentration. The degree of these polynomials would usually be one for bidentate ligands although second-degree polynomials are conceivable.

$$\frac{1}{v} = \frac{K}{V} \left(1 + \frac{f_1[\text{I}]}{g_1[\text{I}]} \right) \frac{1}{[\text{A}]} + \frac{1}{V} \left(1 + \frac{f_2[\text{I}]}{g_2[\text{I}]} \right) \quad (1)$$

Eqn. (1) is the steady-state rate equation where v is the initial reaction rate, $[A]$ is the concentration of substrate and K and V correspond to the Michaelis constant and maximum velocity respectively.

The prediction of slope and intercept effects for inhibition of this type has been fully discussed by Cleland (1963) and the nature of the inhibition (dead-end, partial etc.) and its degree with respect to inhibitor concentration (linear, hyperbolic etc.) described. A replot of slopes and intercepts as a function of inhibitor concentration for the case of bidentate ligands would be expected to be of low degree, parabolic being just possible (corresponding to $[E][I_2]$ where $[E]$ is the equilibrium concentration of active metal) but cubic highly unlikely. We conclude that where addition of a multidentate metal-chelating agent produces an immediate or time-dependent reversible inhibition by an insertion mechanism, then it is possible to study this by the usual methods of steady-state kinetics under initial-rate conditions at equilibrium with inhibitor.

Type II mechanism: the metal ion-chelating agent forms co-ordination complexes with metal ions removing them from the enzyme

This scheme might be thought to apply where a metal ion is held extremely tenaciously by ligands on the enzyme and can only be effectively removed by adding chelating agent in relatively high concentration. Usually the enzyme would only recover activity slowly when, after removal of inhibitor by dialysis, the metal ion is added. This would be especially the case when removal of metal allowed dissociation into subunits to occur or, alternatively, allowed cofactors to be lost.

We now consider the case where metal ions necessary for enzyme activity are progressively removed by adding increasing concentrations of chelating agent so allowing dissociation of the enzyme into subunits; the enzyme activity, presumed to be proportional to enzyme concentration, being measured by adding substrate before inhibitor is added and when the system has reached equilibrium in the presence of known amounts of inhibitor. If the assumption is made that all the metal ions present in the system originate from the enzyme, we have the stoichiometry of eqn. (2):

Here we have a mol of active enzyme dissociating into b_j subunits, E_j^* , which have altered enzyme activity owing to the removal of c mol of metal ion $[M]$ which combine with d mol of inhibitor $[I]$ producing p mol of co-ordination complex $[C]$ at equilibrium.

By assuming that conservation of metal applies

according to $[M_0] = [E] + [C] + [M] = c/a[E_0]$, where $[E]$, $[C]$ and $[M]$ are equilibrium concentrations of active metal, co-ordination complex and free metal, $[E_0]$ being the original concentration of active enzyme and $[M_0]$ the total metal present we have:

$$K_1^m = \frac{\left[\frac{b_1}{a}[E_0]\lambda\right]^{b_1} \left[\frac{b_2}{a}[E_0]\lambda\right]^{b_2} \dots [M]^c}{\{[E_0](1-\lambda)\}^a}$$

$$K_2^n = \frac{\{p/d[I_0]\Delta\}^p}{\{(1-\Delta)[I_0]\}^d [M]^c}$$

where $\lambda = 1 - ([E]/[E_0])$ and $\Delta = 1 - ([I]/[I_0])$, and $[M] = \{c/a[E_0]\lambda - p/d[I_0]\Delta\}$, giving eqn. (3):

$$\lambda = \frac{a}{c[E_0]} \left\{ \left(\frac{K_1^m [E_0] (1-\lambda)^a}{\left(\frac{\lambda}{a}\right)^s \prod_i (b_i)^{b_i}} \right)^{\frac{1}{c}} + \left(\frac{K_1^m K_2^n [E_0] (1-\lambda)^a}{\left(\frac{\lambda}{a}\right)^s \prod_i (b_i)^{b_i}} \right)^{\frac{1}{p}} [I_0]^{\frac{d}{p}} \right\} \quad (3)$$

Here m is the highest common factor of a , b and c , n is the highest common factor of c , d and p ,

$S = \sum_{i=1}^j b_i$, $(1-\Delta)$ is approximately 1.0 and it is assumed

that metal-containing species other than E_0 and C are unimportant.

In our experience, this general case solution usually simplifies to more convenient forms since many of the coefficients and exponents are known or can be determined independently. For instance, it is usually the case that $c = p$ and this gives eqn. (4):

$$\lambda \left[\frac{(\lambda/a)^s \prod_i (b_i)^{b_i}}{(1-\lambda)} \right]^{\frac{1}{c}} = \frac{a K_1^{\frac{m}{c}}}{c[E_0] \left(\frac{c+S-a}{c} \right)} \left[1 + K_2^{\frac{n}{c}} [I_0]^{\frac{d}{c}} \right] \quad (4)$$

A particularly useful form is given when no dissociation into subunits occurs. This is eqn. (5) in which y is the number of metal ions released from the enzyme and x is the ratio of ligand to metal in the co-ordination complex containing only one metal ion/complex.

$$\lambda \left(\frac{\lambda}{1-\lambda} \right)^{\frac{1}{y}} = \frac{K_1^{\frac{1}{y}}}{y[E_0]} (1 + K_2 [I_0]^x) \quad (5)$$

By obtaining λ values at several inhibitor concentrations, it is thus possible to calculate y and x and, knowing $[E_0]$, the values for K_1 and K_2 .

Typical graphs of λ against $[I_0]$ and expressions from eqn. (5) are shown in Figs. 1 and 2.

Fig. 1. Variation of λ with $[I_0]$ calculated from the theoretical function

$$\lambda \left(\frac{\lambda}{1-\lambda} \right)^{1/y} = \frac{K_1^{\frac{1}{2}}}{y[E_0]} (1 + K_2 [I_0]^x) \approx k [I_0]^x$$

(a) Curves for which $y=2, x=3$. (i) $k=1$; (ii) $k=10^{-1}$; (iii) $k=10^{-2}$; (iv) $k=10^{-3}$. — — —, Horizontal inflexion co-ordinate. (b) Curves for which $y=1, x=2$. (i) $k=10$; (ii) $k=1$; (iii) $k=10^{-1}$; (iv) $k=10^{-2}$. The x axis is the horizontal inflexion co-ordinate.

Fig. 2. Data from Fig. 1(a) where $k=1$

The correct equation was

$$\lambda(\lambda/1-\lambda)^{1/y} = k [I_0]^x$$

where $y=2$ and $x=3$ giving the cubic line (ii). The other curves are for (i) $y=1$; (iii) $y=3$; (iv) $y=4$. They show deviation from integer exponent polynomial in I when y is incorrect.

Separating type I from type II mechanisms

Recognizing the correct mechanism will probably be simple at higher inhibitor concentrations by accumulating information immediately the inhibitor is added and also under equilibrium conditions. If double-reciprocal plots are linear and replots of slope and intercept remain linear or at most, parabolic at high inhibitor concentrations, then ligand substitution according to a type I mechanism is operating. If slope and/or intercept replots do not show a simple functional dependence on inhibitor concentration but plots of $\lambda(\lambda/1-\lambda)^{1/y}$ or the appropriate function are well defined functions of inhibitor concentration, then metal ion dissociation is occurring according to a type II mechanism. It may be difficult in practice to assign a mechanism correctly at low inhibitor concentrations and a further graphical method is now described for this purpose.

Graph of λ against $[I_0]$

Usually λ will equal $(1-[E]/[E_0])$ where excess of inhibitor causes complete loss of enzyme activity. The absolute value will range from 0 or a very small value when $[I_0]=0$ to 1.0 when $[I_0]$ is large and the behaviour of the curve is informative at low $[I]$ values.

The condition for an inflexion in this curve is now sought, i.e. a value for the second derivative of λ with respect to $[I_0]$ changing from positive to negative as it passes through zero.

Type I mechanism. Consider eqn. (1) which simplifies in the absence of inhibitor to $1/v = K/V \cdot 1/[A] + 1/V$ since $f_1[I]$ and $f_2[I]$ have no

constant terms. Then $v_1/v_0 = [E]/[E_0] = 1 - \lambda$ yields:

$$\lambda = \frac{Kf_1[I]g_2[I] + [A]g_1[I]f_2[I]}{(K + [A])g_1[I]g_2[I] + Kf_1[I]g_2[I] + [A]g_1[I]f_2[I]}$$

In the case where $f_1[I] = a[I]$, $f_2[I] = b[I]$ and $g_1[I] = g_2[I] = 1.0$, the second derivative of λ with respect to $[I]$ is:

$$\lambda'' = \frac{-2a_0^2 b_0}{b_0 + a_0 [I]^3}$$

There is thus no point of inflexion for the linear non-competitive case. Similarly, this holds for the linear competitive and uncompetitive cases.

Further analysis shows that parabolic inhibition can exhibit inflexions under the following special conditions:

for $f_1[I] = a[I] + b[I]^2$; $f_2[I] = c[I] + d[I]^2$ and $g_1[I] = g_2[I] = 1$.

(i) Competitive inhibition when: $(a^2 - b)/b < [A]/K$; $c = d = 0$.

(ii) Uncompetitive when: $(c^2 - d)/d < K/[A]$; $a = b = 0$.

(iii) Non-competitive when: $(Ka + [A]c)^2 < (Kb + [A]d)(K + [A])$.

Type II mechanism. We seek the conditions under which eqn. (5) has points of inflexion for $[I] \geq 0$. Let $\lambda_0 = \lambda([I] \rightarrow 0)$.

We write:

$$(\lambda/\lambda_0)^{1+y} = (1 - \lambda)\sigma^y \quad (6)$$

where $\sigma[I] = 1 + \beta_1[I] + \beta_2[I]^2 + \dots + \beta_N[I]^N$, β_j being the j th cumulative stability constant.

$$\lambda'' = \frac{\lambda(1 - \lambda)y[\sigma\sigma'' \cdot U^2 - (1 + y)(\sigma')^2]}{\sigma^2 \cdot U^3}$$

where $U = 1 + y - \lambda y$, and primes indicate differentiation with respect to $[I]$.

Inflexions occur where $\lambda'' = 0$ and changes sign. Since $\lambda = 1$ is an asymptote we are left with $\lambda = 0$ and:

$$\sigma\sigma'' \cdot U^2 - (\sigma')^2(1 + y) = 0 \quad (7)$$

$\lambda = 0$ gives an inflexion at those values of I which are roots of $\lambda[I] = 0$, i.e. negative $[I]$.

Inflexions due to eqn. (7), if any, are points where it intersects with the original function eqn. (6).

We now investigate those conditions under which the two intersect. For $\sigma = 1 + \beta_1[I]$ eqn. (7) becomes degenerate (i.e. independent of λ) and hence we conclude that there is no point of inflexion for the case where one chelating molecule binds one metal ion; irrespective of the number of metal ions released by the enzyme.

Further discussion now applies to those cases where $\sigma[I]$ is a polynomial of degree $N \geq 2$.

Eqn. (7) can be solved for λ to give:

$$\lambda = 1 + \frac{1}{y} - \frac{\sqrt{1+y}}{y} \left[\frac{(\sigma')^2}{\sigma\sigma''} \right]^{\frac{1}{2}} \quad (8)$$

As $[I] \rightarrow \infty$:

$$\lambda \rightarrow 1 + \frac{1}{y} - \frac{\sqrt{1+y}}{y} \sqrt{\frac{N}{N-1}}$$

which lies between 0 and 1 for all values of y and of N .

As $I \rightarrow 0$:

$$\lambda \rightarrow 1 + \frac{1}{y} - \frac{\sqrt{1+y}}{y} \sqrt{\frac{\beta_1^2}{2\beta_2}} = 1 + \frac{1}{y} - \frac{\sqrt{1+y}}{y\sqrt{2}} \sqrt{\frac{K_2}{K_3}}$$

This is <0 if $K_2 > 2(y+1)K_3$ (see Fig. 3c) and >0 if $K_2 < 2(y+1)K_3$ (see Figs. 3a and 3b).

Where $\sigma[I]$ has been approximated by $k[I]^x$ as in Fig. 1 the expression eqn. (8) becomes independent of $[I]$ and simplifies to:

$$\lambda = 1 + \frac{1}{y} - \frac{\sqrt{1+y}}{y} \cdot \sqrt{\frac{x}{x-1}}$$

giving points of inflexion on a horizontal line, e.g. 0.44 for $y = 2$, $x = 3$ as in Fig. 1(a). Specifically when $y = 1$, $x = 2$ this is the horizontal axis as in Fig. 1(b).

Sigmoid curves as a function of $[I]$

From the foregoing we conclude that sigmoid curves are not possible for type I mechanisms except under special circumstances for parabolic inhibition.

For type II mechanisms sigmoid curves are not possible for $x = 1$ and any value of y .

Otherwise sigmoid curves are possible as illustrated in Fig. 3. In practice, since $\sigma[I]$ will often be of the type $k[I]^x$, this means that sigmoid curves will usually be given except for $x = 2$, $y = 1$ as well as $x = 1$ and all y as above.

Alternative processes

It is possible that more involved processes take place than the straightforward dissociation postulated above.

For example, sequential release of metal ions from the enzyme or sequential binding of metal ion with ligand might occur.

Consequently, we investigate as a specific case the following scheme:

 Fig. 3. Points of inflexion in curves of λ against $[I_0]$

In each case an inflexion occurs at the intersection of $(\lambda/\lambda_0)^{1+y} = (1-\lambda)\sigma[I_0]^y$ and

$$\lambda = \frac{1+y}{y} - \frac{\sqrt{1+y}}{y} \left[\frac{(\sigma')^2}{\sigma\sigma''} \right]^{\frac{1}{2}}$$

In each graph:

$$\text{Point A} = \frac{1+y}{y} - \frac{\sqrt{1+y}}{y} \left(\frac{K_2}{2K_3} \right)^{\frac{1}{2}}$$

$$\text{Line B} = \frac{1+y}{y} - \frac{\sqrt{1+y}}{y} \left(\frac{N}{N-1} \right)^{\frac{1}{2}}; 0 \leq B \leq 1$$

$$\text{Line C} = \left[1 - \frac{v_1(\Pi = \infty)}{v_0(\Pi = 0)} \right]$$

In (a) $A > 1$; (b) $0 < A < B$; (c) $A < 0$.

The various concentrations are:

$$[EM_2] = [E_0](1-\lambda); [M] = (2-\alpha)[E_0]\lambda - [I_0]\Delta;$$

$$[EM_1] = \alpha[E_0]\lambda; [MI] = a_1[I_0]\Delta;$$

$$[E] = (1-\alpha)[E_0]\lambda; [MI_2] = a_2[I_0]\Delta;$$

$$[I] = [I_0](1-\Delta); [MI_3] = a_3[I_0]\Delta;$$

where $0 \leq \alpha \leq 1$, $a_1 + a_2 + a_3 = 1$.

Then

$$(2-\alpha)[E_0]\lambda - [I_0]\Delta = \frac{(1-\lambda)}{\alpha\lambda} K_{11} = \frac{\alpha K_{12}}{1-\alpha} = \frac{a_1}{K_{21}} \frac{\Delta}{1-\Delta} \quad (9)$$

and $K_{22} = a_2/a_1[I_0](1-\Delta)$, $K_{23} = a_3/a_2[I_0](1-\Delta)$. By using the approximation $1-\Delta \sim 1$ as before we find:

$$a_1(1 + K_{22}[I_0] + K_{22}K_{23}[I_0]^2) = 1$$

and so:

$$(2-\alpha)[E_0]\lambda = [I_0]\Delta + \frac{a_1[I_0]\Delta}{K_{21}[I_0]} = [I_0]\Delta \cdot \frac{\sigma[I_0]}{\sigma[I_0] - 1}$$

therefore

$$(2-\alpha)[E_0]\lambda = \frac{\alpha}{1-\alpha} \cdot K_{12} \sigma[I_0]$$

on substitution for $[I_0]\Delta$ from eqn. (9):

$$\text{i.e.} \quad \frac{(1-\alpha)(2-\alpha)}{\alpha} \cdot E_0 \lambda = K_{12} \cdot \sigma[I_0] \quad (10)$$

Also from eqn. (9):

$$\frac{(1-\lambda)K_{11}}{\lambda K_{12}} = \frac{\alpha^2}{1-\alpha} = q$$

giving $\alpha = -q + \sqrt{q^2 + 4q}/2$

and therefore:

$$\frac{(1-\alpha)(2-\alpha)}{\alpha} = \frac{\sqrt{q^2 + 4q}(q+2 - \sqrt{q^2 + 4q})}{2q}$$

which $\sim 2/\sqrt{q}$ for $q \ll 4$

Then:

$$\lambda \left(\frac{\lambda}{1-\lambda} \right)^{\frac{1}{2}} = \frac{\sqrt{K_{11}K_{12}}}{2[E_0]} \{\sigma[I_0]\}$$

Table 1. *A comparison of the approximate equation for $y = 2$, $x = 3$ as derived from eqn. (5) with the exact equation*

Good agreement is given between the approximate and exact equations as long as the degree of dissociation of metalloenzyme is small. For details see the text.

q	$f(q)$	$2/\sqrt{q}$	α
1.0	0.8	2.0	0.618
10^{-1}	4.673	6.324	0.2701
10^{-2}	18.12	20.0	0.095
10^{-4}	198.01	200.0	0.01

which compares with the approximate equation derived from eqn. (5), if we let $y = 2$, $x = 3$:

$$\lambda \left(\frac{\lambda}{1-\lambda} \right)^{\frac{1}{2}} = \frac{\sqrt{K_1}}{2[E_0]} (1 + K_2[I_0]^3)$$

It is useful to compare the error introduced by approximating:

$$\frac{\sqrt{q^2 + 4q(q+2 - \sqrt{q^2 + 4q})}}{2q} \quad \text{by} \quad \frac{2}{\sqrt{q}}$$

and the limitations placed on α by different values of q . This is shown in Table 1 from which it can be seen that as long as $K_{11}/K_{12} < 10^{-2}$ and $\alpha < 0.1$ the approximation is good except that $(1 + K_2[I_0]^3)$ is replaced by $\sigma[I_0]$.

We have also analysed other mechanisms and obtained exact solutions. In general, accounting for metal in intermediate metal-enzyme complexes is

difficult unless the cumulative stability constant is well defined, i.e. for:

$k_{1j} \gg K_{1(j-1)}$, for all j values. Accounting for metal bound up in intermediate metal-ligand complexes means that $\{1 + K_2[I_0]^3\}$ is replaced by $\sigma[I_0]$. Since in practice the cumulative stability constant for the ligand in question will be well defined this will be a valid approximation. The error involved in equating $(1 - \Delta)$ and 1.0 is usually minimal. If this approximation is not made then the exact equation can be retrieved by replacing $[I_0]$ by $[I_0] \times \epsilon$ (where $\epsilon = [I_0]\Delta$), in eqn. (6).

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